GENE EXPRESSION PROFILE IN THE MIDDLE CEREBRAL ARTERY AND FRONTAL CORTEX OF HYPERTENSIVE RABBITS

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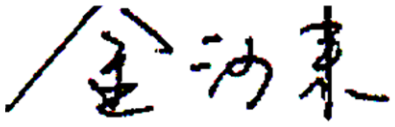
Declaration

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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SUMMARY

Hypertension is known to contribute to the progression of plaque formation in hyperlipidemia and is an important risk factor in cerebral atherosclerosis and stroke. This study presents an expression profile of various genes that are involved and regulated in hypertension in both intra- and extracranial vessels. The expression of genes and extension of damage to cerebral arteries caused by hypertension were evaluated quantitatively and morphologically in 10 New Zealand White rabbits with and without hypertension, induced using the 2-kidney, 1-clip Goldblatt hypertension model. Genes in the frontal cortex and middle cerebral artery respectively were filtered from microarray analysis and subjected to the Ingenuity Pathway Analysis where canonical pathways and a network of other genes related to our gene input were generated. From the selection of our genes of interest, 8 genes PPARA, PRL-R, PTGDR, P450, Gab3, Tnfs14, Sell and Lass3 were verified by RT-PCR.These genes have shown to be involved in the progression or contribution towards inflammatory diseases such as atherosclerosis. PPARA is a major regulator of lipid metabolism. Gab3 and Tnfs14 produce cytokines and chemokines while P450 protein is known to increase metabolism of arachidonic acid, a precusor in the production of eicosanoids which involves PTGDR. In addition, Tnfs14 and Sell are involved in endothelial cell adhesion, activation and disruption. Endothelial dysfunction is a hallmark for vascular diseases, and is often regarded as a key early event in the

development of atherosclerosis thus further study of these genes may lead to a better understanding on the role of hypertension in stroke.

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Abbreviations

1K1C	one kidney one clip
2K1C	two kidney one clip
ASTN2	adaptor-related protein complex 1, astrotactin 2
CACNA1B	calcium channel, voltage-dependent, N type, alpha 1B subunit
CALB1	thrombin, cerebellar calbindin
CBF	cerebral blood flow
CRB1	crumbs homolog 1 (Drosophila)
CYP1A2	Cytochrome P450 1A2
DNASE1L3	deoxyribonuclease I-like 3
FAM167A	family with sequence similarity 167, member A
FOXN1	forkhead box N1
Gab3	Growth factor receptor bound protein 2-associated protein 3
GCLC	glutamate-cysteine ligase
HTN	Hypertension
ICA	internal carotid artery
IPA	Ingenuity Pathway Analysis
LASS3	LAG1 homolog, ceramide synthase 3
MCA	middle cerebral artery

MMP1	Interstitial collagenase Precursor
NZW	New Zealand wild type
ODZ4	odd Oz/ten-m homolog 4
PCGEM	parametric test based on cross gene error model
PCR	Polymerase chain reaction
PENK	proenkephalin
PPARA	peroxisome proliferator-activated receptor alpha, partial
Prlr	prolactin receptor (partial)
PTGDR	Prostanoid DP receptor
PVDF	polyvinylidene difluoride
PYY	peptide YY
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SELL	L-selectin
TAC1	neurokinin A
TCRB	T-cell receptor beta-chain V9, partial cds
TNF	tumor necrosis factor
TNFSF14	tumor necrosis factor (ligand) superfamily, member 14
TNFSF15	tumor necrosis factor (ligand) superfamily, member 15
WHO	World Health Organization

Chapter 1: Introduction

1. 1 Stroke

Stroke has been defined by the World Health Organization (WHO) as “rapidly developing clinical signs of focal or global disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death with no apparent cause other than of vascular origin” (Miller, 1999). Based on the analysis of American Heart Association, stroke becomes the leading cause of morbidity and mortality, especially in the elderly. Its incidence and prevalence increase sharply with age that 72% of the subjects suffering a stroke are over age 65. Many types of stroke are identified, such as ischemic stroke, intracerebral haemorrhage, subarachinoid haemorrhage and cerebral venous sinus thrombosis, but regardless of type, surviving a stroke could have devastating impact that the patients can experience loss of vision, speech, paralysis and confusion, physical and mental disabilities, depending on the part of brain that is affected. Therefore stroke brings a substantial economical burden on individuals and society.

As described by WHO, stroke is a problem of vascular origin (eg. hypertension). In addition, lifestyle such as smoking, high salt intake, and underlying heart disease, diabetes, hyperlipidemia, family history, prior stroke or transient

ischemic attack, blood clotting disorders have also been shown to be the risk factors of stroke.

Among them, high blood pressure is one of the highest contributing risk factor accounting for 91% of stroke incidents followed by high level of cholesterol (78%) and smoking (77%). (Travis *et al.*, 2003; Horst and Korf, 1997)

Ischemic stroke is caused by transient or permanent reduction in cerebral blood flow (CBF), resulting in the deficiency of glucose and oxygen supply to the territory of the affected region (Barber et al, 2003; Zemke *et al*, 2004,). As ischemic stroke is by far the most common type of stroke, accounts for 70 to 80% of total stroke incidences (Feigin et al, 2003), of which 60% are attributable to large artery ischemia, developing effective ischemic stroke therapies has been the main goal for many researchers. The effort of development has led to several important successes during the past decade, though many disappointing failures.

1.2 Hypertension

Hypertension (HTN) or high blood pressure is a cardiac chronic medical condition in which the systemic arterial blood pressure is elevated. Persistent hypertension is one of the risk factors for stroke, myocardial infarction, heart failure and arterial aneurysm, and is a leading cause of chronic kidney failure(Miksche et al, 1970; Ninomiya et al, 2011). Moderate elevation of arterial

blood pressure leads to shortened life expectancy. Dietary and lifestyle changes can benefit blood pressure control and decrease the risk of associated health complications, although drug treatment may prove necessary in patients for whom lifestyle changes prove ineffective or insufficient.

The most prevalent hypertension type is essential hypertension, affecting 90–95% of hypertensive patients (Carretero & Oparil 2000). The direct cause of essential hypertension is unknown but there are many factors such as sedentary lifestyle (Kyrou *et al.* 2006), stress and obesity (Wofford & Hall 2004) that may contribute to the risk. Another risk factor is an increased level of renin that is secreted by the kidney (Segura & Ruilope 2007). Hypertension also increases the hardening of arteries (Riccioni 2009), leading to heart disease, peripheral vascular disease (Singer & Kite 2008) and strokes (White 2009).

Hypertension can cause significant adaptive changes in the cerebral circulation (Strandgaard & Paulson 1995). The role of hypertension, atherosclerosis, and inflammation of blood vessels as the leading causes of stroke have been well established (Ross 1993, Lawes *et al.* 2004). Both atherosclerosis and hypertension are two important pathological vascular processes that involve an altered vascular homeostasis characterized by endothelial dysfunction. Furthermore, prospective cohort studies have shown that the association between blood pressure and risk of stroke was continuous and log linear (Lewington *et al.* 2002, 1995, Lawes *et al.* 2003). Although hypertension alone does not induce atherosclerosis, experimental studies of animals have shown to accelerate plaque

formation and progression (Hollander *et al.* 1993, Xu *et al.* 1991), where the extent and severity of cerebral atherosclerosis were significantly related to the severity of hypertension in one study and plaque formation was still significantly greater in a hypertensive group of animals despite marked lowering of serum cholesterol values in another study.

What makes hypertension in particular such an aggressive target for treatment is that it is the most important modifiable risk factor for ischemic stroke (Sacco *et al.* 1997). Many randomized clinical trials of antihypertensive drugs have demonstrated both a reduction of carotid intima-media thickness, a validated measure of subclinical atherosclerosis and predictor risk for clinical cardiovascular events, than a protection against clinical stroke events. Large body of evidences has shown that antihypertensive drugs exert important anti-atherosclerotic effects in non-cerebral vessels, which depend to some extent on the degree of blood pressure lowering provided by these drugs(Riccioni 2009). However little is known about the biochemical and molecular features of the impact of hypertension in cerebral vessels.

1.3 Middle Cerebral Artery

The middle cerebral artery (MCA) is one of the three major paired arteries that supply blood to the cerebrum. The MCA arises from the internal carotid and

continues into the lateral sulcus where it then branches and projects to many parts of the lateral cerebral cortex (Zhao BQ et al, 2001). It also supplies blood to the anterior temporal lobes and the insular cortices.

The left and right MCAs rise from trifurcations of the internal carotid arteries and thus are connected to the anterior cerebral arteries and the posterior ecommunicating arteries, which connect to the posterior cerebral arteries(Yanni rt al, 2004).

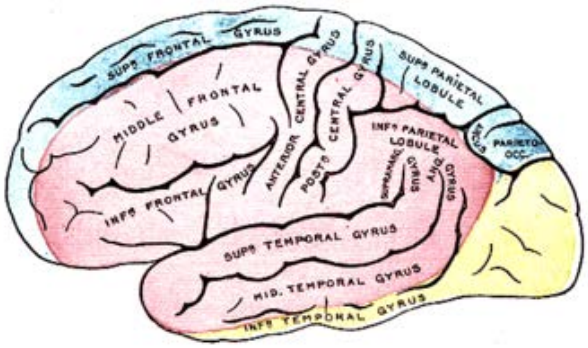

Figure A, Outer surface of cerebral hemisphere, showing areas supplied by cerebral arteries.

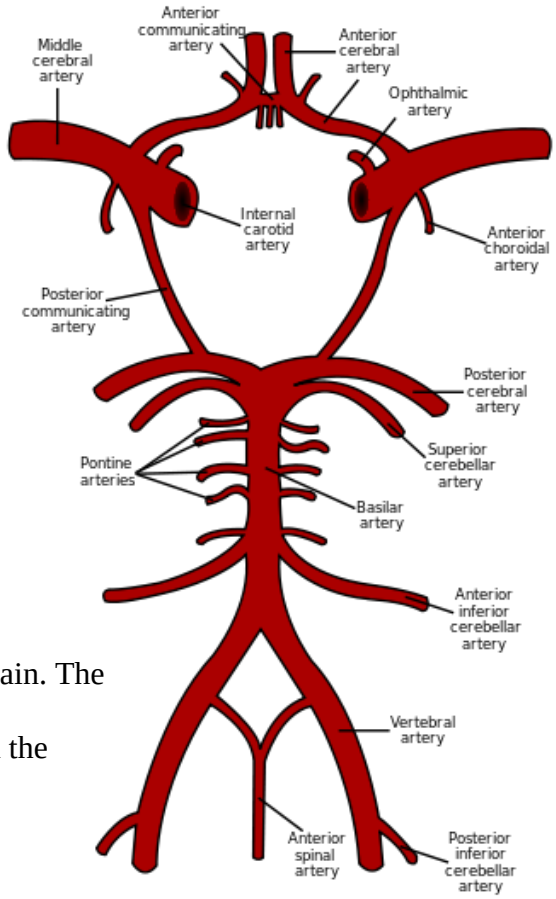

Figure B, The arterial circle and arteries of the brain. The middle cerebral arteries (top of figure) arise from the internal carotid arteries.

(Figure A and B were both adopted from Rhcastilhos, *Gray's Anatomy,2007*)

Middle cerebral artery stroke describes the sudden onset of focal neurologic deficit resulting from brain infarction or ischemia in the territory supplied by the middle cerebral artery (MCA).

The MCA is by far the largest cerebral artery and is the vessel most commonly affected by cerebrovascular accident (CVA). The MCA supplies most of the outer convex brain surface, nearly all the basal ganglia, and the posterior and anterior internal capsules. Infarcts that occur within the vast distribution of this vessel lead to diverse neurologic sequelae. Understanding these neurologic deficits and their correlation to specific MCA territories has long been researched.

Research has also focused on the correlation between specific neurologic deficits after MCA stroke and differing outcomes and prognoses (Brown et al, 2010). Such efforts are important in ascertaining who may benefit from emergent antithrombotic therapies. Furthermore, these research efforts may later allow physiatrists to target rehabilitative efforts more effectively in appropriately selected patients who may derive benefit.

1.4 Frontal Cortex

The frontal cortex is an area in the brain of mammals, located at the front of each cerebral hemisphere and positioned anterior to (in front of) the parietal lobe and superior and anterior to the temporal lobes. It is separated from the parietal lobe

by a space between tissues called the central sulcus, and from the temporal lobe by a deep fold called the lateral (Sylvian) sulcus (Chen ZZ et al, 2009). The precentral gyrus, forming the posterior border of the frontal lobe, contains the primary motor cortex, which controls voluntary movements of specific body parts.

The frontal lobe contains most of the dopamine-sensitive neurons in the cerebral cortex. The dopamine system is associated with reward, attention, short-term memory tasks, planning, and motivation (Lamchak et al, 2002). Dopamine tends to limit and select sensory information arriving from the thalamus to the fore-brain.

1.5 Animal Model of Hypertension

Much of the understanding of the molecular mechanisms involved in the pathophysiology of the cardiovascular system has been gained from in vitro studies. Nevertheless, the role of specific gene products in cardiovascular homeostasis should also be clarified in intact animals. Molecular biology, in particular, genetically modified animals generated by transgenic technology, has been used for investigating the basic mechanism of gene regulation and creating models for human diseases (Robbins et al, 1993).

Small animal models including rats and mice are being used to study the effects of hypertension. The current standard animal model that is widely used in related studies is two kidney one clip (2K1C) model or one kidney one clip model(1K1C), which carries a new understanding of the mechanisms in the end-organ damage so that could provide new avenues for prevention of cardiovascular events. Many studies have examined effects of hypertension in gene expression changes in tissues such as liver, but thus far little is known about changes in the intracranial vessels and brain.

Since the original work of Goldblatt et al (Goldblatt, 1934), the 2K1C (two kidney one clip) and 1K1C (one kidney one clip) animal models have greatly contributed to our knowledge of cardiovascular diseases. In the 2K1C model, one renal artery is constricted to chronically reduce renal perfusion, and the other kidney remains untouched. In the 1K1C model, one kidney is removed, and the other undergoes artery.

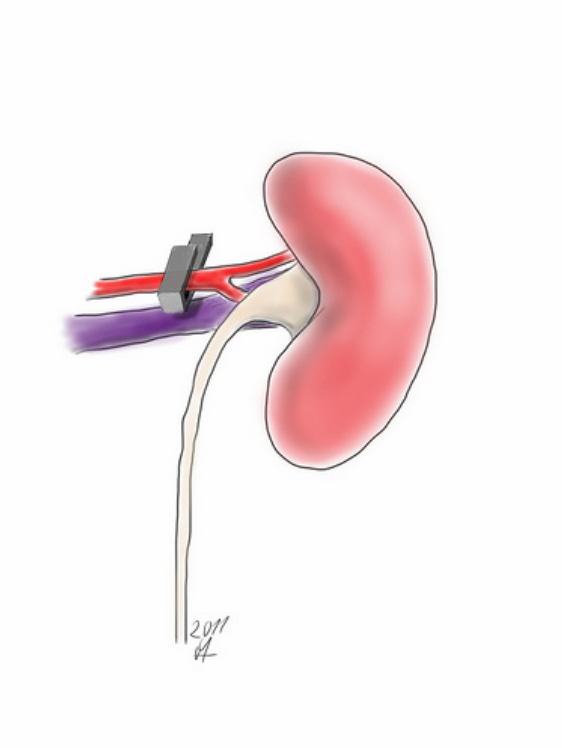

Figure C, Quick concept for the two kidneys, one clip model of renal hypertension (Adopted from Michael Hultström, *Discussing kidney physiology, nephrology and science, with interludes for dogs, photography, judo, dogs and food, 2001*)

In both models, the earliest phase of hypertension is characterized by a rapid rise in plasma renin in response to low renal arterial pressure and by the consequent increase in circulating Ang II. However, the mechanisms of the chronic phase of hypertension differ between the two models. In the 2K1C model, hypertension is maintained by a continuously activated renin-angiotensin system because pressure diuresis of the contralateral normal kidney prevents hypervolemia. In contrast, volume retention by the single stenotic kidney of the 1K1C animal shuts off renin secretion, providing a model of low-renin, volume-dependent hypertension. Nevertheless, both models develop cardiovascular hypertrophy constriction (De Simone et al, 1993; Corbier A et al, 1994).

Pathophysiology of renovascular hypertension

	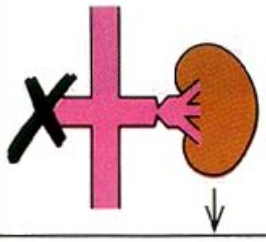	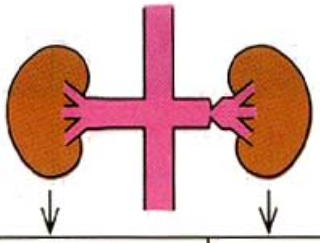	
Renin content of kidney	No change	Decreases on contralateral side	Increases on stenotic side
Blood pressure	Significantly increases	Significantly increases	
Plasma renin activity	No change or decreases	Significantly increases	
Plasma volume	Increases	No change	
Blood pressure after block of angiotensin II	No change	Decreases	

Table A. Although hypertension is equally present in both models, the one-kidney model demonstrates normal to low plasma renin activity low renin content in the kidney, and increased plasma volume; the two-kidney model demonstrates increased renin in the plasma and clipped kidney as well as reduced or absent renin in the unclipped kidney. The hypertension of the two-kidney model can be normalized with an angiotensin II antagonist, however, the hypertension of the one-kidney model does not respond to such treatment. (Adopted from Laragh et al, 2003)

Animal models of ischemic stroke have usually failed to successfully transition into human clinical practice. This is especially the case with mouse and rat models which have been the most commonly used models but have never made a successful transition into human application (Donnan, 2008). Larger animals apparently are required, but the selection is limited by other factors including unfavorable anatomy. The rabbit model is the one exception to this and has been successful in tPA therapy development leading to tPA as the standard of care in human stroke. (Bednar et al, 1997; Hamilton et al, 1994; Hoyte 2004)However, most rabbit models show wide variability in the strokes thus limiting precision. They use relatively short survival of a few hours to two days while deaths and severe symptoms limit longer term studies (Reasoner et al, 1996; Maynard et al, 1998; Jahan et al, 2008).

Adult New Zealand rabbits are large enough to provide adequate arterial detail to mimic human anatomy. A modified technique (Culp et al, 2007; Caldwell et al, 2008; Kirchhof et al, 2002) of angiographic selection of the internal carotid artery (ICA) through femoral artery access with subsequent single clot embolization allowed us to produce similar strokes in a series of rabbits with a low death rate. This allows the study of stroke location and its relation to neurological function deficits. And this is an important step towards refinement and further validation of the animal model and can lead to its future use in long term comparison of new therapies.

1.6 Aim of the study

This study aims to examine the effect of hypertension alone in cerebral vessels, largely in the middle cerebral artery and frontal cortex and provide an overview on the genes that are regulated even before the onset of atherosclerosis in brain that will eventually lead to stroke. Early recognition or detection of genes regulated in this process could thus be made potentially relevant in a clinical setting or for pharmaceutical intervention in future.

The present study was carried out in NZW rabbits in view of the importance of hypertension in neurological disorders such as stroke and vascular dementia, gene expression changes implicated in hypertension and its downstream impact in the vessels and brain.

Chapter 2: Materials and Methods

2.1 Rabbits and treatment

Ten male New Zealand White rabbits weighing between 2-2.5kg were fed normal rabbit diet pellet and water ad libitum. After an acclimatisation period of 2 weeks, rabbits were divided into hypertension (2K1C) and control groups (2K1CC). Hypertension was produced by constricting the left renal artery with a silver clip 0f 0.6 mm internal diameter. In the 2K1CC group, sham surgery was performed on the left renal artery. The right kidney was not touched in both groups.

Rabbits were anesthetized by ketamine (70mg/kg weight). The kidney was exposed through a small flank incision, externalized, and carefully maintained with an ophthalmic chalazion forceps. For clipping, the renal artery of the left kidney was individualized over a short segment by blunt dissection, and a clip was placed close to the aorta. The kidney was then gently pushed back into the retroperitoneal cavity. For right nephrectomy, two ligatures were passed around the renal vascular pedicle and ureter and were tied. The kidney was removed without the adrenal gland. The muscle layer was sutured, and the skin incision was closed with surgical staples. A sham procedure, which included the entire surgery with the exception of artery clipping, was applied in control.

Mean arterial pressure (MAP) of the rabbit was measured via the central ear artery (Powerlab 4/30, ADInstruments, USA) and blood from both groups were collected at 0, 4, 10 and 12 weeks. Approximately 3ml of blood was withdrawn from the rabbit ear artery and collected into 6ml BD Vacutainer® Serum Tubes

with Clot activator and silicone-coated interior (BD Franklin Lakes, NJ). Rabbits were deeply anaesthetized intra-muscularly with 0.2ml/kg ketamine/xylazine cocktail prior to blood drawing and euthanasia by intravenous injection of 1ml pentabarbitol (300mg/ml) at the end of 12 weeks. The brain was carefully removed and the middle cerebral artery (MCA), frontal cortex (FC) and hippocampus (HC) from the right brain was manually dissected and immersed in RNAlater® (Ambion, TX, USA), snap frozen in liquid nitrogen and stored in -80^{o}C till further analysis. The left brain, aorta, liver and kidneys were fixed in two changes of 4% paraformaldehyde and stored at 4^{o}C till further analysis. All procedures performed were approved by the Institutional Animal Care and Use Committee of the National University of Singapore in accordance with the National Advisory Committee for Laboratory Animal Research Guidelines.

2.2 Serum Cholesterol

The Cholesterol/Cholesteryl Ester Quantitation Kit provides a simple method for sensitive quantification of free cholesterol, cholesteryl esters, or both by colorimetric or fluorometric methods. Majority of the cholesterol in blood is in the form of cholesteryl esters which can be hydrolyzed to cholesterol by cholesterol esterase. Cholesterol is then oxidized by cholesterol oxidase to yield H2O2which reacts with a sensitive cholesterol probe to produce color (λmax =570 nm) and fluorescence (Ex/Em = 535/587 nm). The assay detects total

cholesterol (cholesterol and cholesteryl esters) in the presence of cholesterol esterase or free cholesterol in the absence of cholesterol esterase in the reaction. Cholesteryl ester can be determined by subtracting the value of free cholesterol from the total (cholesterol plus cholesteryl esters).

Whole blood was centrifuged at 1000 x g for 15 min and the serum was transferred to new vials and kept frozen in -80^{o}C till further analysis. Serum cholesterol levels were measured by fluorometric assay (Ex/Em 535/587 nm) according to the standard cholesterol kit instructions (BioVision, Inc., SF, USA). Samples were ran in triplicates and were read with a microplate reader (Infinite® i-control, Tecan Trading AG, Switzerland).

2.3 RNA extraction

The RNA extraction procedure combines the selective binding properties of a silica-based membrane with the speed of microspin technology. Nucleic acids, either DNA or RNA, are adsorbed onto the silica-gel membrane in the presence of chaotropic salts, which remove water from hydrated molecules in solution. Polysaccharides and proteins do not adsorb and are removed. A specialized high-salt buffer system allows upto 100 μg of RNA longer than 200 bases to bind to the silica membrane.

Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to a spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. After a wash step, pure nucleic acids are eluted under low- or no-salt conditions in small volumes.

Total RNA was extracted and isolated from MCA and FC using TRizol reagent (Invitrogen, CA, USA) according to the manufacturer's recommended protocol. The lysate was homogenized, then centrifuged for 30s at 14000g in a microfuge and the supernatant was mixed with 650 µl of 70 % ethanol to clear lysate. The sample was applied to an RNeasy mini spin column (silicagel membrane, maximum binding capacity is 100 µg of RNA longer than 200 bases) and spun for 30 sec at 14000g and then flow-through was discarded. The RNA bound to the membrane was washed with buffer RW1 and RPE sequentially. High-quality RNA was then eluted in 20 µl of RNase free water. The concentration and purity of the extracted RNA was evaluated spectrophotometrically at 260 and 280 nm (Biophotometer, Eppendorf, Germany). The RNA samples were stored at -80° C until experiments.

2.4 cDNA Synthesis

The extracted RNA was purified and reverse transcribed with the RNeasy® Mini Kit (Qiagen, Inc., CA, USA) and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) respectively. Reaction conditions were 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 s. cDNA thus obtained was then diluted in sterile water and stored at -20° C.

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2.5 Microarray Analysis

Labelled cRNA from purified MCA and FC mRNA of 2K1C and 2K1CC rabbits was hybridized to the 1-colour Agilent Rabbit Microarray (Agilent, G2519F-020908), according to the manufacturer's recommended protocol. 10ul of total RNA was submitted to Genomax Technologies, Singapore, where RNA quality was analyzed using an Agilent 2100 Bioanalyzer, and cRNA generated and labelled using the one-cycle target labelling method. cRNA generated from each sample was hybridized to a single array according to standard Agilent protocols. Data collected were exported into GeneSpring v11 (Agilent Technologies, CA, USA) software for analysis using parametric test based on cross gene error model (PCGEM). Unpaired *t*-test approach was used to identify differentially expressed genes (DEGs).

2.6 Pathway and network analyses

The gene sets were analyzed using the Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Mountain View, CA). The respective up- and down-regulated DEGs from the treated and control samples containing gene identifiers and corresponding expression values was uploaded into IPA application. Each identifier mapped to its corresponding object in Ingenuity's Knowledge Base (*p-value> 0.05* cut-off of >4 or 10 fold change) was set to identify molecules whose expression was significantly differentially regulated. Canonical pathways analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) a ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed. 2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

2.7 Real-Time PCR

Real-time PCR amplification was performed on the 7500 Real time PCR system to validate the expression of common genes of interest between the MCA and FC using TaqMan® Universal PCR Master Mix and customised rabbit probes. The PCR conditions were initial incubation of 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were carried out in triplicate. The fold change for each gene expression in MCA and FC was analysed and calculated by using the $2^{-\Delta\Delta C_T}$ method as described by Livak and Schmittgen. Rabbit beta-actin (Oc03824857_g1) was used as housekeeping genes. Unavailable rabbit primers were designed based on the sequences provided by the National Center for Biotechnology Information database.

Table B: gene selection for RT-PCR

	Gene	Gene symbol	Fold Change in FC	Fold Change in MCA	Related Disease	Network
Highest Up Reg Fold change in FC	Cytochrome P450 1A2	CYP1A2	24.38	6.09	hypertrophy, weight gain, infarction	NW1 (FC)/ NW2 (common)
	peroxisome proliferator-activated receptor alpha, partial	PPARA	16.62	3.00	hypertension, coronary artery disease, Alzheimer's disease	NW1 (FC)/ NW2 (common)
	T-cell receptor beta-chain V9, partial cds	TCRB	15.21	2.84	atherosclerosis	NW1 (FC)/ NW2 (common)
Common Up Reg Fold change in FC	crumbs homolog 1 (Drosophila)	CRB1	10.29	3.73	coronary artery disease	NW2 (FC)/NW1(Common)
	tumor necrosis factor (ligand) superfamily, member 14	TNFSF14	8.65	2.52		NW1 (FC)/NW2(Common)
	Growth factor receptor bound protein 2-associated protein 3	Gab3	8.42	2.28	Alzheimer's disease	NW2 (FC)/NW1(Common)
Common Up Reg Fold Change in MCA	family with sequence similarity 167, member A	FAM167A	6.68	14.04	Alzheimer's disease, insulin-dependent diabetes mellitus	NW6(common)
	LAG1 homolog, ceramide synthase 3	LASS3	5.16	11.44	non-insulin-dependent diabetes mellitus	NW7 (Common)
	deoxyribonuclease I-like 3	DNASE1L3	2.72	8.27	liver neoplasia, liver cancer, hepatocellular carcinoma, cancer atherosclerosis,	NW1 (common)
	Interstitial collagenase Precursor	MMP1	2.90	8.12	Alzheimer's disease, cardiovascular disorder, inflammatory disorder	NW2 (common)
FC net work 1	tumor necrosis factor	TNF	7.16			NW1 (FC)/ NW2 (common)
	prolactin receptor (partial)	Prlr	8.10			NW1(FC)
	peptide YY	PYY	10.15			NW1(FC)
	L-selectin	SELL	8.32			NW1(FC)
	Prostanoid DP receptor	PTGDR	7.39			NW1(FC)
	tumor necrosis factor (ligand) superfamily, member 15	TNFSF15	7.34			NW1(FC)

2.8 Western Blot

Frontal cortex from NZW rabbits were used for this portion of the study. A portion of the FC was homogenized in 10 volumes of ice-cold lysis buffer (150 mM sodium chloride, 50 mM Tris–hydrochloride, 0.25 mM EDTA, 1% Triton X-100, 0.1% sodium orthovanadate, and 0.1% protease inhibitor cocktail, pH 7.4). After centrifugation at 10,000*g* for 10 min at 4°C, the supernatant was collected. The protein concentrations in the preparation were then measured using the Bio-Rad protein assay kit. The homogenates (20 μg) were resolved in 10% SDS–polyacrylamide gels under reducing conditions and electrotransferred to a polyvinylidene difluoride (PVDF) membrane. Non-specific binding sites on the PVDF membrane were blocked by incubating with 5% non-fat milk in 0.1% Tween 20 TBS (TTBS) for 1 h. The PVDF membrane was then incubated overnight in polyclonal antibody to PTGDR, PPARA, PRL-R, P450, Gab3, Tnfs14, SELL and Lass3 in 3% bovine serum albumin in TBST (Table C). After washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated secondary anti-mouse or anti-goat (Pierce, Rockford, IL) for 1 h at room temperature. Immunoreactivity was visualized using a chemiluminescent substrate (Supersignal West Pico, Pierce, and Rockford, IL). Loading controls were carried out by incubating the blots at 50 °C for 30 min with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–hydrochloride, pH 6.7), followed by reprobing with a mouse monoclonal antibody to β-actin (Sigma; diluted 1:10,000 in TBST) and horseradish peroxidase-conjugated anti-mouse IgG (1:2,000 in TBST, Pierce). Exposed films containing blots were

scanned, and the densities of the bands were measured, using Gel-Pro Analyzer 3.1 program (Media Cybernetics, Silver Spring, MD). The densities of the bands were normalized against those of β-actin, and the mean ratios were calculated. Possible significant differences between the values from the 2K1C rabbits and control rabbits were then analyzed, using Student's *t*-test. $P < 0.05$ was considered significant

Table C: **Concentration of primary and secondary antibodies used for western blots**

Antibodies	Source	Primary (antibody dilution)	Secondary (antibody dilution)
Mouse monoclonal to LIGHT(ab57901)	Abcam	1:200	1:2000
Goat polyclonal to Cytochrome P450 1A1+1A2 (ab4227)	Abcam	1:100	1:2000
PRL-R(B10):sc-74520	Santa Cruz	1:200	1:2000
PPARA(C-20):sc-1982	Santa Cruz	1:200	1:2000
Gab3(D-20):sc-22615	Santa Cruz	1:200	1:2000
LASS3(T-17):sc-55962	Santa Cruz	1:200	1:2000
DP(S-14):sc-55818	Santa Cruz	1:500	1:2000
L-Selectin(N-18):sc-6946	Santa Cruz	1:100	1:2000

Chapter 3: Results

3.1 Body Weight

The average body weight between the two groups was not found to be significantly different (Figure 1A). At the end of 12 weeks, mean weight were 3.37 kg and 3.46 kg for the 2K1C and 2K1CC groups respectively.

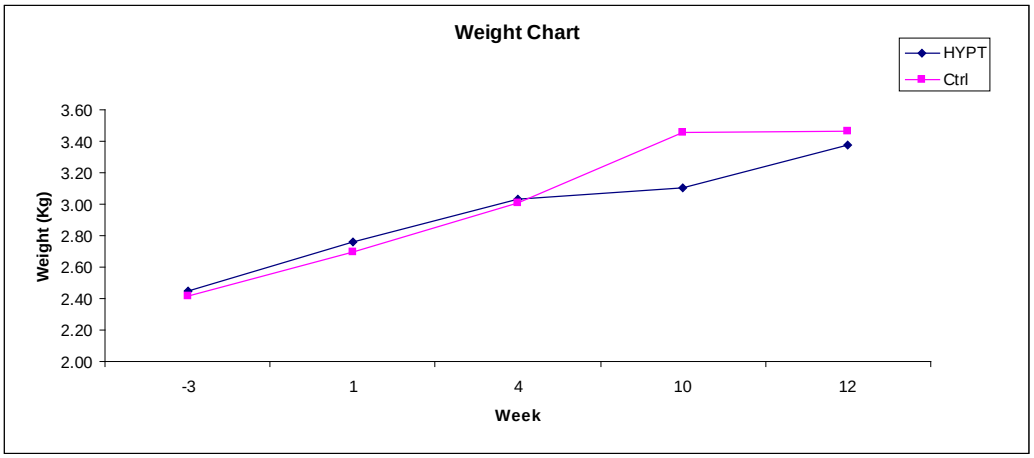
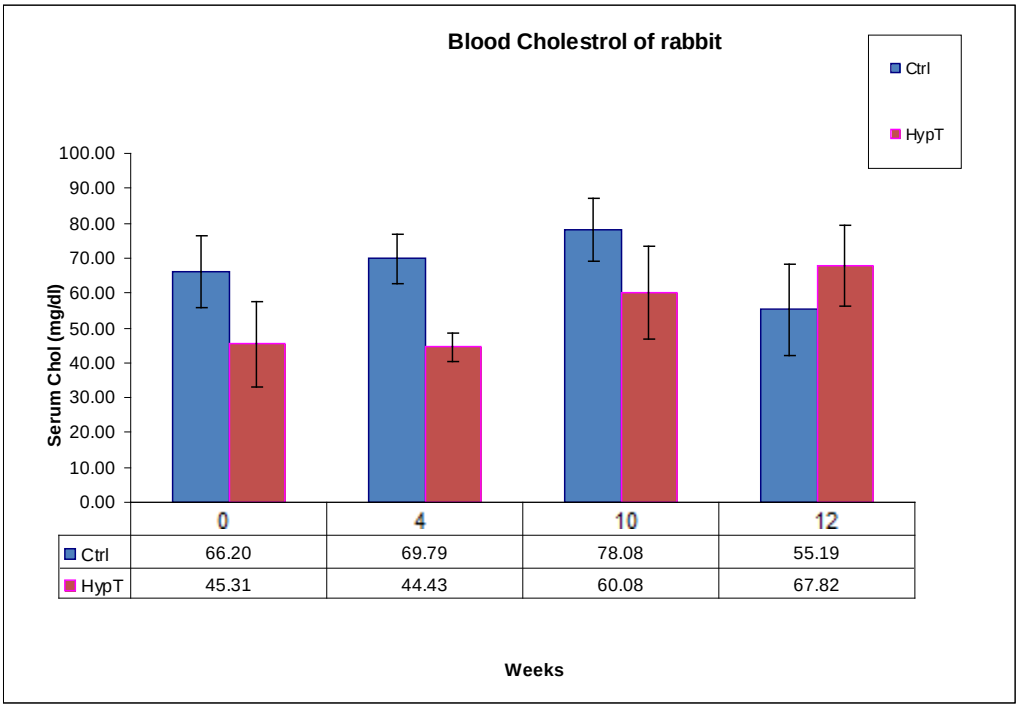


Fig 1. A) Weight chart of rabbits measured on alternate weeks during the study. Sample size n=10. No significant differences between the HYPT and Ctrl group.

3.2 Serum Cholesterol and Mean Arterial Pressure

No significant increase in total cholesterol was observed in the serum of 2K1C rabbits at 0, 4, 10 and 12 weeks compared to the normal fed rabbits (Figure 1B). Before initiation of the diet, the mean cholesterol level of treated rabbits was 45.31mg/dl while control animals had a mean of 66.20mg/dl. However, after 12 weeks of cholesterol feeding, the mean cholesterol level of treated rabbits increased to 67.82 while control animals were 55.19mg/dl.



	0	4	10	12
Ctrl	66.20	69.79	78.08	55.19
HypT	45.31	44.43	60.08	67.82

Fig1. B) Serum cholesterol levels in rabbits measured at baseline, 4, 10 and 12 weeks. Data are plotted as mean ± S.D. and analyzed by Student's T-test. *P* < 0.05 indicates significant differences.

The mean arterial pressure (MAP) at the different time points within the 2K1C group was significantly increased from baseline at 85mmHg, with a peak of 166.5mmHg at 10 weeks. In comparison, the baseline of 2K1CC group was 69.8mmHg and it increased to 106.4mmHg at 10 weeks (Figure 1C).

Fig1.B)

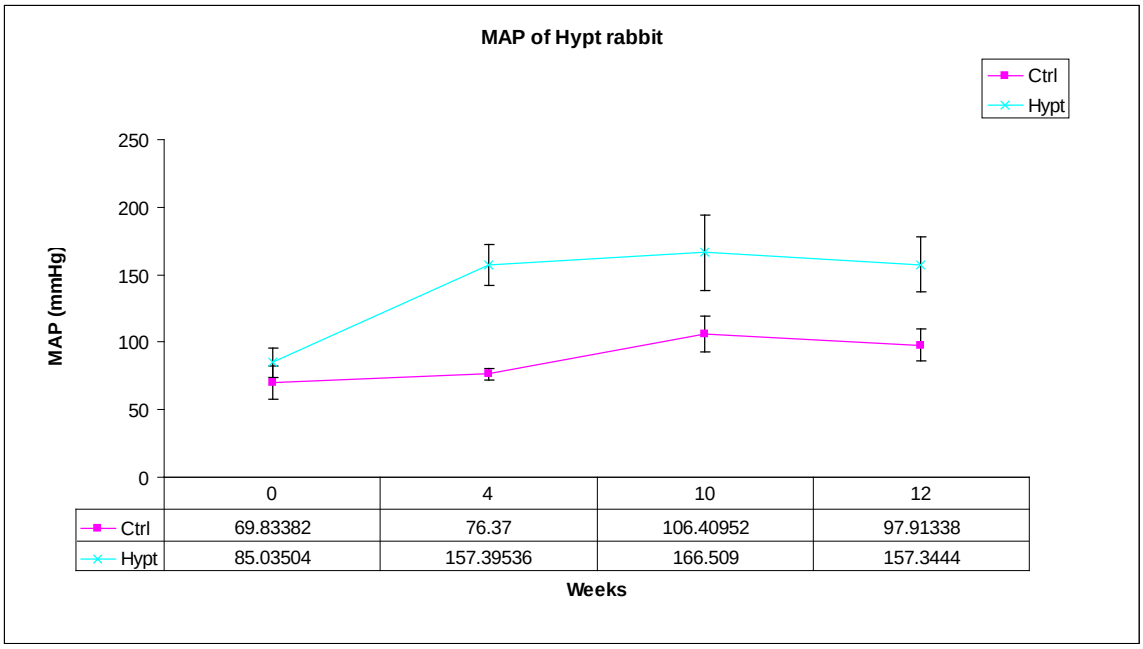


Fig1.C) Mean arterial pressure (MAP) levels in rabbits measured at baseline, 4, 10 and 12 weeks. Data are plotted as mean ± S.D. and analyzed by Student's T-test. *P* < 0.05 indicates significant differences. Significant difference began from Week 4.

3.3 Microarray data collection and analysis

The 2K1C and 2K1CC rabbits were sacrificed 12 weeks after the surgery was initiated and MCA and FC were harvested for microarray analysis. The gene expression profile on the FC and MCA of the 2K1C group was compared to the 2K1CC group. A total of 10440 and 12106 genes were found for FC and MCA respectively. A total of 854 and 248 genes which had greater than 4-fold change were found in the FC and MCA respectively. Common genes were then identified between the two brain regions and a total of 195 genes with greater than 4-fold change were found (Fig 2). Of these, unknown and repeated genes were omitted and only up regulated genes with more than 7 fold change and down regulated genes with more than 4 fold changes in the FC and up- and down regulated genes with more than 6 fold changes in the MCA were analyzed. The results were then classified using Ingenuity Pathway Analysis (IPA).

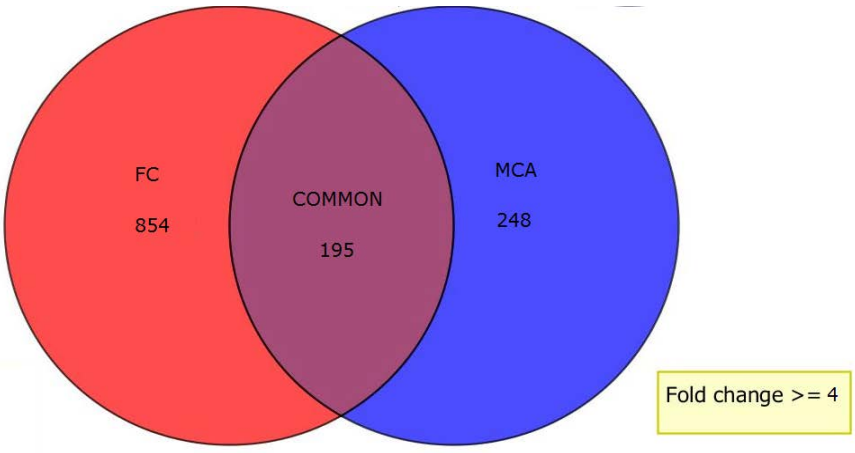


Fig 2. Venn diagram summarizing genes with *p*>0.05 and fold change >4 expressed in middle cerebral artery and frontal cortex.

3.3.1 Differentially expressed genes found in MCA

There were a total of 29 up regulated DEGs (differential expressed genes) and 31 down regulated DEGs identified in the MCA (Table 1, 2). DEGs that were found up-regulated include family with sequence similarity 167, member A (FAM167A), substance P, neuropeptide gamma, neurokinin A (TAC1), thrombin, cerebellar calbindin (CALB1) and proenkephalin (PENK). Down regulated genes included forkhead box N1 (FOXN1), BRCA2 and CDKN1A interacting protein and secreted frizzled-related protein 4 (SFRP4).

Table 1. Up Regulated genes in MCA with more than 6 fold change

Gene	Gene symbol	Fold Change	*P*-value
family with sequence similarity 167, member A	FAM167A	14.04	0.00000
substance P, neuropeptide gamma, neurokinin A	TAC1	13.08	0.01093
LAG1 homolog, ceramide synthase 3	LASS3	11.44	0.00027
thrombin mRNA	THROMBIN	10.16	0.00145
family with sequence similarity 53, member C	Fam53c	9.87	0.00001
cerebellar calbindin	Calb1	8.96	0.00515
proenkephalin	PENK	8.73	0.01095
Type II adenylyl cyclase Fragment	LOC100009201	8.29	0.00001
deoxyribonuclease I-like 3	DNASE1L3	8.27	0.01184

tubulin tyrosine ligase-like family, member 5	TTLL5	8.16	0.00004
olfactory receptor, family 1, subfamily J, member 1	OR1J1	8.14	0.00006
Interstitial collagenase Precursor (Matrix metalloproteinase-1)(MMP-1)	MMP1	8.12	0.00047
TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor	TAF15	7.45	0.00008
Purkinje cell protein 4	PCP4	7.19	0.00438
zinc finger, DHHC-type containing 23	ZDHHC23	7.18	0.00001
odz, odd Oz/ten-m homolog 4 (Drosophila)	ODZ4	7.16	0.00003
ankyrin and armadillo repeat containing	ANKAR	7.05	0.00003
tyrosine hydroxylase mRNA, partial cds	BDNF	6.88	0.00122
stefin A2 mRNA, partial cds	Stfa2	6.69	0.01085
N(alpha)-acetyltransferase 25, NatB auxiliary subunit	NAA25	6.43	0.00049
corticotropin releasing hormone binding protein	CRHBP	6.38	0.00009
ankyrin repeat and SOCS box-containing 4	Asb4	6.36	0.00000
solute carrier family 5 (sodium/glucose cotransporter), member 12	SLC5A12	6.34	0.00104
Gonadotropin-releasing hormone receptor	GNRHR	6.33	0.00003
secretagogin, EF-hand calcium binding protein	SCGN	6.28	0.01846
Cytochrome P450 1A2	CYP1A2	6.15	0.00034
KIAA0232	KIAA0232	6.13	0.00007
Fanconi anemia, complementation group C	FANCC	6.10	0.00005
chromosome X open reading frame 57	CXorf57	6.07	0.03688

Table 2. Down Regulated genes in MCA with more than 6 fold change

Gene	Gene symbol	Fold Change	*P*-value
forkhead box N1	Foxn1	26.20	0.00529
coiled-coil domain containing 55	CCDC55	24.13	0.00008
BRCA2 and CDKN1A interacting protein	CDKN1A	23.79	0.00001
THUMP domain containing 3	THUMPD3	17.18	0.00002
AT rich interactive domain 2 (ARID, RFX-like)	Arid2	16.92	0.00002
Probable methyltransferase C20orf7, mitochondrial Precursor	C20orf7	15.92	0.00001
ADAM metallopeptidase with thrombospondin type 1 motif, 17	Adamts17	15.85	0.00010
peptidylprolyl isomerase G (cyclophilin G)	ppig	12.61	0.00007
hematopoietic prostaglandin D synthase	Hpgds	12.38	0.00612
cyclin H	CCNH	10.89	0.00002
Lyrm7 homolog (mouse)	LYRM7	10.41	0.00003
male-specific lethal 2 homolog (Drosophila)	MSL2	9.72	0.00082
secreted frizzled-related protein 4	SFRP4	9.32	0.00001
ankyrin 2, neuronal	ANK2	9.32	0.00019
Myosin heavy chain Fragment	Mhc	9.27	0.00003
Ras-related protein Rab-7a	Rab7a	9.20	0.00013
guanine deaminase	GDA	8.64	0.03019
NLR family, pyrin domain containing 5	NLRP5	8.60	0.00001
membrane-spanning 4-domains, subfamily A, member 1	Ms4a2	7.86	0.00057

sorting nexin 9	SNX9	7.82	0.00101
protein tyrosine phosphatase, non-receptor type 23	PTPN22	7.80	0.00123
large subunit GTPase 1 homolog (S. cerevisiae)	LSG1	7.69	0.00010
coiled-coil domain containing 59	Ccdc59	7.66	0.00001
ribosomal protein S12	RpS12	7.57	0.00364
breast carcinoma amplified sequence 2	BCAS2	7.39	0.00027
mitochondrial ribosomal protein L15	mRpL15	6.97	0.00000
Transmembrane protein C3orf1 (Protein M5-14)	C3orf1	6.72	0.00217
olfactory receptor, family 4, subfamily D, member 2	OR1D2	6.61	0.00151
tumor necrosis factor, alpha-induced protein 8	TNFAIP8	6.59	0.00194
LIM domains containing 1	LIMCH1	6.10	0.00001
Neurolysin, mitochondrial Precursor	NLN	6.05	0.00332

3.3.2 Differentially expressed genes found in FC

There were a total of 36 up regulated DEGs and 13 down regulated DEGs identified in the FC (Table 3,4). DEGs that were up-regulated include Cytochrome P450 1A2 (CYP1A2), odz, odd Oz/ten-m homolog 4 (ODZ4), peroxisome proliferator-activated receptor alpha, partial (PPARA), and tumor necrosis factor (TNF). Down regulated DEGs included calcium channel, voltage-dependent, N type, alpha 1B subunit (CACNA1B), glutamate-cysteine ligase (GCLC), adaptor-related protein complex 1, astrotactin 2 (ASTN2), sodium channel, voltage-gated, type III, alpha subunit (SCN3A), and sigma 2 subunit (AP1S2).

Table 3. Up Regulated genes in FC with more than 7 fold change

Gene	Gene symbol	Fold Change	*P*-value
Cytochrome P450 1A2	CYP1A2	24.38	0.00009
odz, odd Oz/ten-m homolog 4 (Drosophila)	ODZ4	19.13	0.00063
peroxisome proliferator-activated receptor alpha, partial	PPARA	16.62	0.00003
T-cell receptor beta-chain V9, partial cds	TCRB	15.21	0.00008
keratin 32	KRT32	12.35	0.00006
olfactory receptor, family 1, subfamily J, member 1	OR1J1	11.56	0.00447
transmembrane protease, serine 11A	TMPRSS11A	11.41	0.03971
leucine rich repeat containing 53	LRRC53	10.69	0.01634
double homeobox A	DUXA	10.41	0.00007
crumbs homolog 1 (Drosophila)	CRB1	10.29	0.00000
Peptide YY	PYY	10.15	0.00031
transcription factor CP2-like 1	Tcfcp2l1	9.61	0.00050
pre-alpha S2a casein	CSN1S2A	9.37	0.03325
CU464574 SSH library 4 cells embryo subtracted from morulae embryo		9.25	0.03452
glycine receptor, alpha 1	GLRA1	9.18	0.04313
haloacid dehalogenase-like hydrolase domain containing 3	HDHD3	9.16	0.01265
tumor necrosis factor (ligand) superfamily, member 14	TNFSF14	8.65	0.00005
Rabbit K+		8.44	0.00142

Growth factor receptor bound protein 2-associated protein 3	Gab3	8.42	0.00039
selectin L	SELL	8.32	0.04840
prolactin receptor	Prlr	8.10	0.00272
methyltransferase like 11B	Mettl11b	8.03	0.00061
Connexin 50 Fragment	Gja8	7.93	0.00005
T-cell receptor delta chain V2-D-J-C, partial cds		7.92	0.01044
interleukin 9	Il9r	7.66	0.00026
trans-2,3-enoyl-CoA reductase-like	Tecrl	7.61	0.00104
Alpha-3 type IV collagen Fragment	Col4a3bp	7.55	0.04080
PHD and ring finger domains 1	UHRF1	7.47	0.00713
prostaglandin D2 receptor	PTGDR	7.39	0.00169
beta-carotene 15,15'-monooxygenase 1	BCMO1	7.39	0.00025
protease, serine, 33	PRSS33	7.35	0.00002
tumor necrosis factor (ligand) superfamily, member 15	TNFSF15	7.34	0.00058
peptidyl arginine deiminase, type IV		7.31	0.00342
tumor necrosis factor	TNF	7.16	0.00392
purinergic receptor P2Y, G-protein coupled, 10	Padi4	7.08	0.01065
Putative uncharacterized protein C11orf80	P2ry1	7.07	0.00037

Table 4. Down Regulated genes in FC with more than 4 fold change

Gene	Gene symbol	Fold Change	*P*-value
ubiquinol-cytochrome c reductase binding protein	UQCRB	15.94	0.03329
immunoglobulin superfamily, member 9	IGSF9	11.40	0.04205
FGGY carbohydrate kinase domain containing	FGGY	7.90	0.02627
SEC24 family, member B (S. cerevisiae)	SEC24B	6.21	0.04005
REV1 homolog (S. cerevisiae)	REV1	5.98	0.01982
calcium channel, voltage-dependent, N type, alpha 1B subunit (CACNA1B)	Cacna1b	5.47	0.01625
glutamate-cysteine ligase, catalytic subunit	Gclc	5.38	0.02757
DENN/MADD domain containing 5A	Dennd5a	4.92	0.04442
forkhead box F2	FOXF2	4.85	0.01935
astrotactin 2	ASTN2	4.80	0.01031
sodium channel, voltage-gated, type III, alpha subunit	Scn3a	4.56	0.00422
WD repeat domain 61	Wdr61	4.42	0.04084
adaptor-related protein complex 1, sigma 2 subunit	AP1S2	4.22	0.04349

3.3.3 Differentially expressed genes found in common

There were 11 up regulated DEGs according to the highest fold changes of the FC and 8 up regulated DEGs according to the highest fold changes of the MCA (fold change >8) (Table 5a,b) that were found in common between the FC and MCA. These included CYP1A2, ODZ4, PPARA, CRB1 and MMP1, some of which are important genes found associated with AD, diabetes and CAD. There were 24 down regulated DEGs that were in common in both the FC and MCA (fold change >2) (Table 6) however not much is known about many of these genes or they did not seem to be associated with any form of diseases.

Table 5a. Up Regulated genes in common with more than 8 fold change (FC)

Gene	Gene symbol	Fold Change in MCA	*P*-value	Fold Change in FC	*P*-value
Cytochrome P450 1A2	CYP1A2	6.09	0.00006	24.38	0.00009
odz, odd Oz/ten-m homolog 4	ODZ4	4.86	0.00042	19.13	0.00063
peroxisome proliferator-activated receptor alpha, partial	PPARA	3.00	0.02050	16.62	0.00003
T-cell receptor beta-chain V9, partial cds	TCRB	2.84	0.01261	15.21	0.00008
olfactory receptor, family 1, subfamily J, member 1	OR1J1	5.27	0.00155	11.56	0.00447
leucine rich repeat containing 53	LRRC53	2.38	0.00538	10.69	0.01634
crumbs homolog 1 (Drosophila)	CRB1	3.73	0.00133	10.29	0.00000
haloacid dehalogenase-like hydrolase domain containing 3	HDHD3	3.82	0.00021	9.16	0.01265
tumor necrosis factor (ligand) superfamily, member 14	TNFSF14	2.52	0.01107	8.65	0.00005
Oryctolagus cuniculus K+		4.23	0.00000	8.44	0.00142
Growth factor receptor bound protein 2-associated protein 3	Gab3	2.28	0.00361	8.42	0.00039

Table 5b. Up Regulated genes in common with more than 8 fold change (MCA)

Gene	Gene symbol	Fold Change in MCA	*P*-value	Fold Change in FC	*P*-value
family with sequence similarity 167, member A	FAM167A	14.04	0.00000	6.68	0.00092
LAG1 homolog, ceramide synthase 3	LASS3	11.44	0.00027	5.16	0.00151
family with sequence similarity 53, member C	Fam53c	9.87	0.00001	4.54	0.00049
Type II adenylyl cyclase Fragment	LOC100009201	8.29	0.00001	4.27	0.00001
deoxyribonuclease I-like 3	DNASE1L3	8.27	0.01184	2.72	0.02320
tubulin tyrosine ligase-like family, member 5	TTLL5	8.16	0.00004	3.96	0.00020
olfactory receptor, family 1, subfamily J, member 1	OR1J1	8.14	0.00006	6.75	0.00540
Interstitial collagenase Precursor	MMP1	8.12	0.00047	2.90	0.00021

Table 6. Down Regulated genes in common with more than 2 fold change

Gene	Gene symbol	Fold Change in MCA	*P*-value	Fold Change in FC	*P*-value
immunoglobulin superfamily, member 9	IGSF9	2.79	0.04124	11.40	0.04205
REV1 homolog (S. cerevisiae)	REV1	4.14	0.04045	5.98	0.01982
delta-like 4 (Drosophila)	DLL4	2.50	0.03809	3.60	0.02787
beta tropomyosin	TPM2	2.30	0.00462	3.41	0.00358
cell division cycle 27 homolog (S. cerevisiae)	CDC27	2.46	0.01430	3.31	0.00096
RNA pseudouridylate synthase domain containing 4	RPUSD4	4.11	0.00095	3.23	0.02484
kinesin family member 20A	KIF20A	2.21	0.00620	2.83	0.03375
LIM domains containing 1	LIMCH1	6.10	0.00001	2.83	0.01483
OTU domain containing 6A	OTUD6A	3.59	0.00030	2.64	0.00718
polymerase (DNA directed), alpha 1, catalytic subunit	POLA1	2.23	0.01108	2.61	0.01035
potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	KCNN2	2.01	0.01518	2.61	0.01391
StAR-related lipid transfer (START) domain	STARD5	2.40	0.00092	2.54	0.03710
RNA pseudouridylate synthase domain containing 4	RPUSD4	2.88	0.03351	2.50	0.02324
calcium binding and coiled-coil domain 2	CALCOCO2	2.52	0.00287	2.47	0.00946

guanylate cyclase 1, soluble, 3	GUCY1B3	2.52	0.00344	2.47	0.01127
Glycine N-methyltransferase Fragment	GNMT	4.17	0.00037	2.40	0.04815
transmembrane protein 59	TMEM59L	3.42	0.02361	2.36	0.01426
sperm specific antigen 2	SSFA2	2.57	0.00124	2.33	0.01040
dual specificity phosphatase 1	Dusp1	2.79	0.02360	2.31	0.02954
family with sequence similarity 177, member A1	FAM177A1	4.41	0.00000	2.27	0.04698
tetraspanin 12	TSPAN12	2.09	0.04706	2.25	0.00411
cell division cycle 37 homolog (S. cerevisiae)-like 1	CDC7	2.44	0.00358	2.22	0.03243
Transmembrane protein C3orf1 (Protein M5-14)	C3orf1	6.72	0.00217	2.22	0.03441
Heterogeneous nuclear ribonucleoprotein C (hnRNP C)	HNRNPC	3.29	0.00001	2.20	0.04392

3.4 Pathway and network analyses

The panel of genes significantly correlated with resistance or sensitivity ($P < 0.05$) to the MEK inhibitor was imported into IPA (Ingenuity Systems, http://www.ingenuity.com) to analyze network interactions. Networks of these significantly correlated genes were then algorithmically generated on the basis of their connectivity.

Genes with significant changes in expression following hypertension were assigned to different canonical signaling pathways and subjected to IPA where the resulting 95 DEGs in MCA, 67 DEGs in FC and 59 DEGs in common were mapped to networks defined by the IPA database.

The top network with the highest number of DEGs in MCA is involved in Cell Cycle, Connective Tissue Development and Function, Cell Death. There were a total of 9 networks mapped from the 67 DEGs found in MCA (Figure 3).The top network in FC is involved in Carbohydrate Metabolism, Cell Death, Cellular Assembly and Organization with 16 DEGs involved. There were a total of 13 networks mapped 67 DEGs in FC (Figure 4). There were 9 networks found from the common DEGs between MCA and FC (Figure 5). The top network had 16 DEGs and was functionally involved in Cell Cycle, Cell Death, Cell-To-Cell Signaling and Interaction.

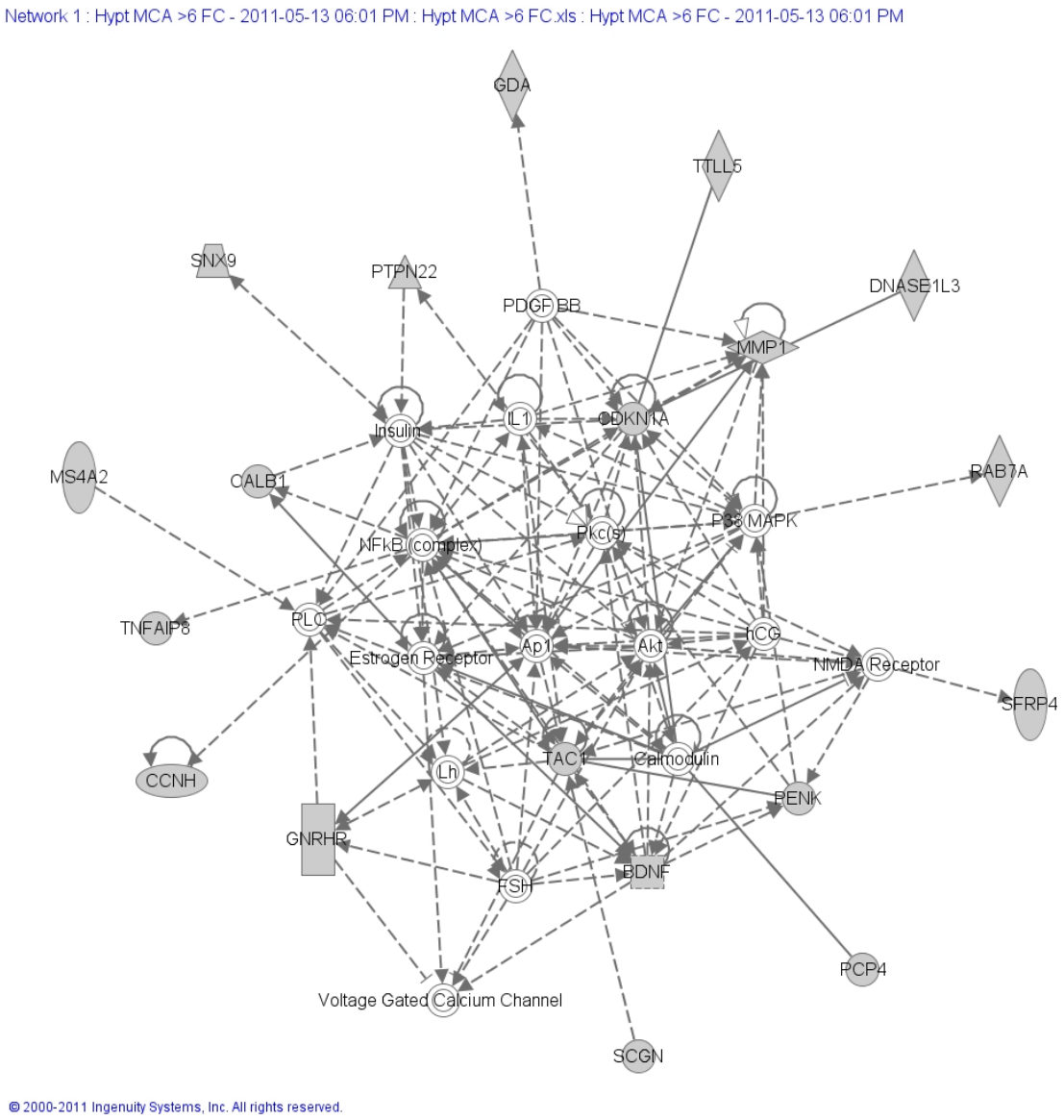


Fig 3. Network of genes mapped in middle cerebral artery

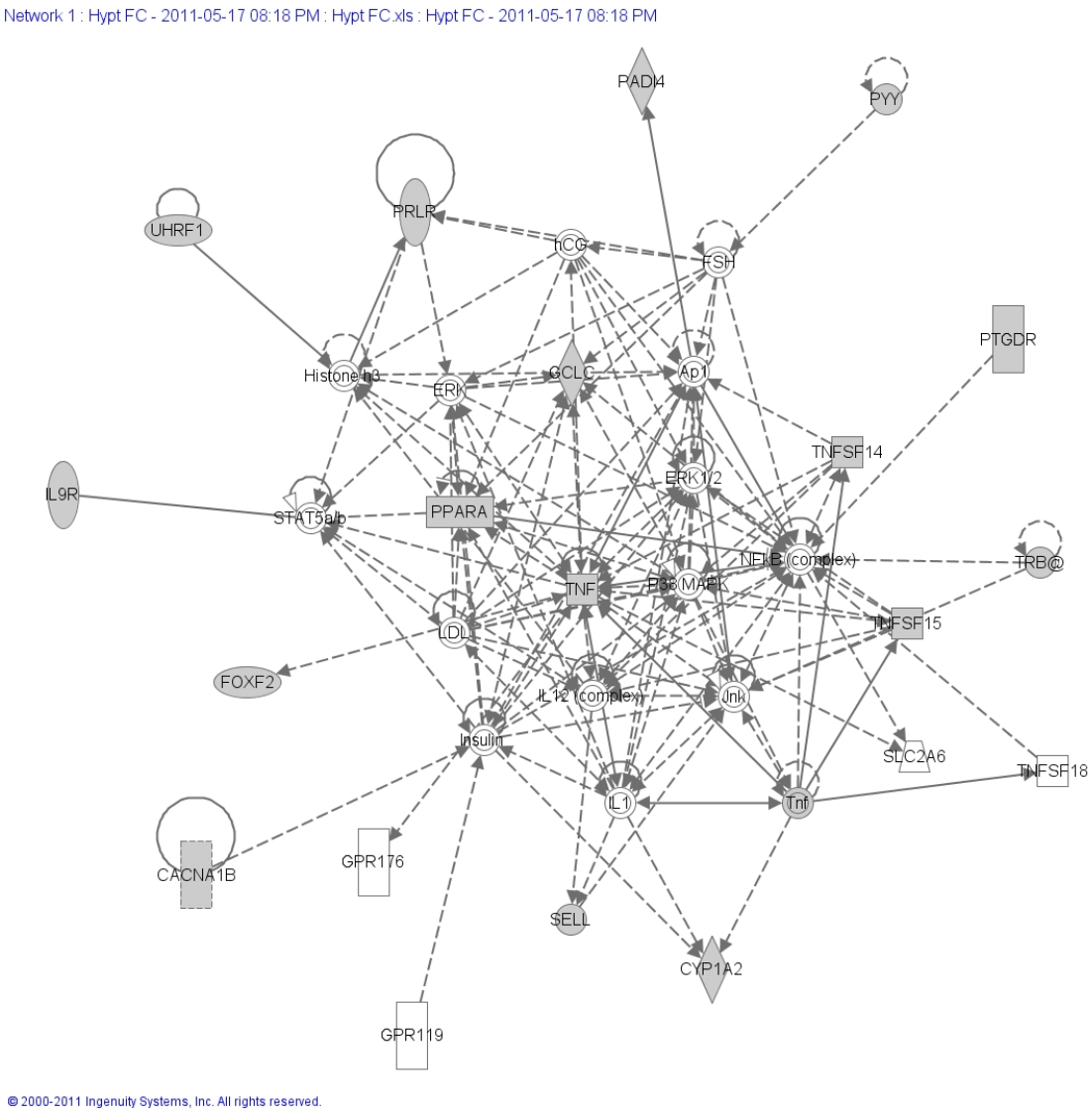


Fig 4. Network of genes mapped in frontal cortex

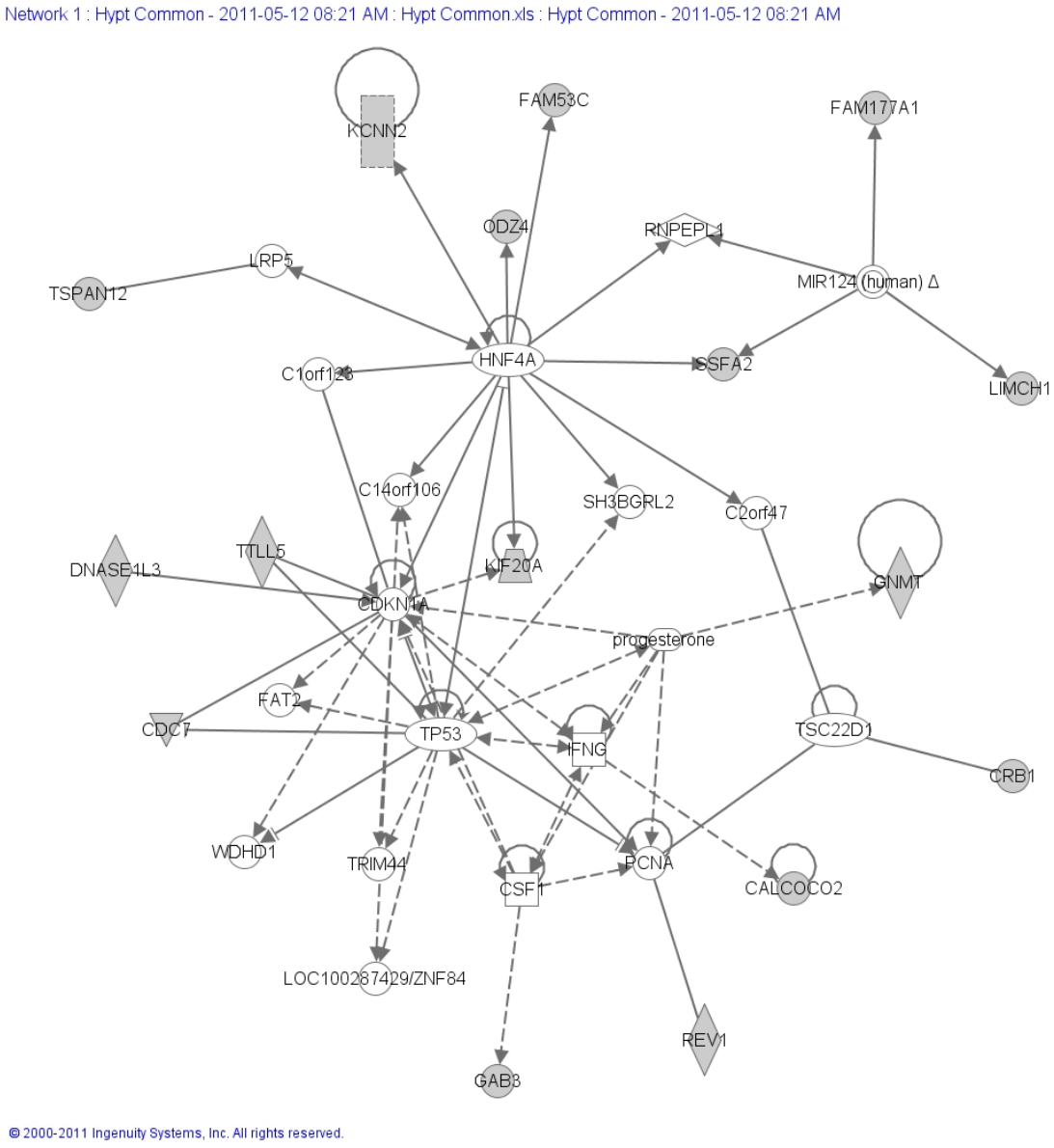


Fig 5. Network of common genes mapped in middle cerebral artery and frontal cortex

3.5 Real-Time PCR

Real-time RT-PCR was used to validate the results of the microarray analysis for the selected genes of interest. In total, 16 top DEGs were selected to be detected by this method. According to the results (Figure 6), there were 8 genes that were verified. PTGDR, PPARA and P450 showed up regulation while Gab3, PRLR, Tnfs14, SELL and Lass3 showed down regulation.

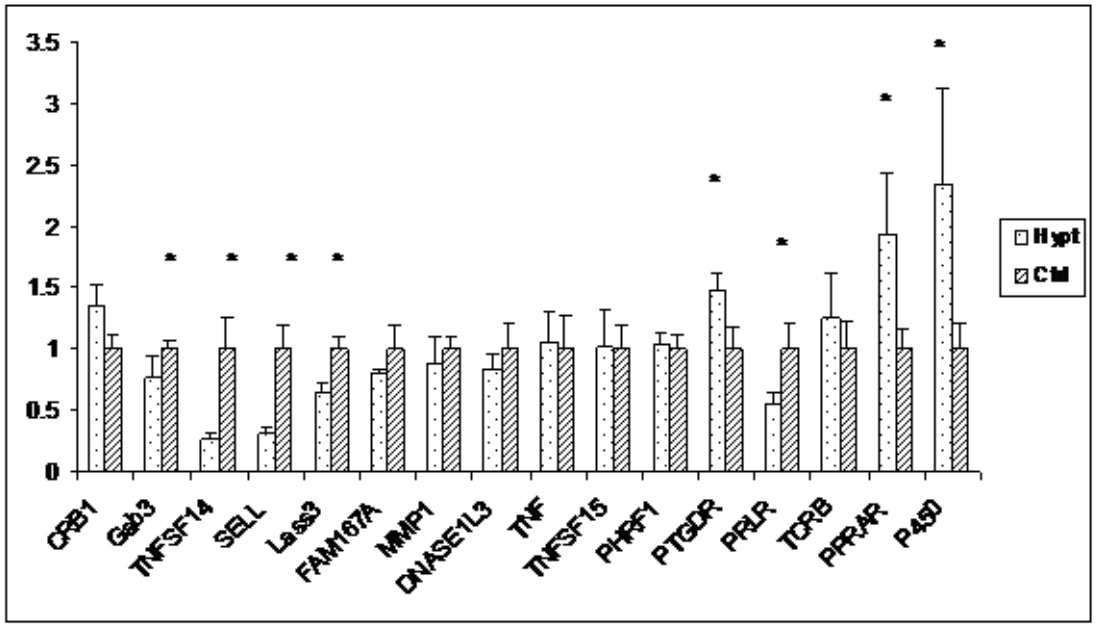


Fig 6. Real-Time PCR results. Data are plotted as mean ± S.D. and analyzed by Student's T-test. *P* < 0.05 indicates significant differences.

3.6 Western Blot

The antibodies to Gab3, LASS3, Tnfsf14, PTGDR, SELL, PPARA, PRLR and P450 detected bands at around 75kDa, 46kDa, 25kDa, 43kDa, 81kDa 55kDa, 100kDa and 56kDa respectively, consistent with the expected molecular weights of these proteins. The antibody to β-actin reacted against a band at 42 kDa. Significant difference in the density ratios of P450, PPARA, LASS3 and PTGDR to β-actin bands was observed in western blots of the 2K1C rabbits, compared to the control rabbits at this time (Fig 7a, b). Among the four genes with significant change, P450, PPARA and PTGDR show upregulation to the control group while LASS3 show downregulating function. All of them are in accordance with the results from the Real Time PCR. Besides, two of the above genes, Gab3 and Tnfsf14, do have significant difference between the treatment group and the control group, however, both of them expressed higher than the control samples, which is just the opposite from the previous analysis. Contrastively, although PRLR and SELL, the last two genes had the same trend of decrease according to former results, their regulation ability was not as strong as the group with significance.

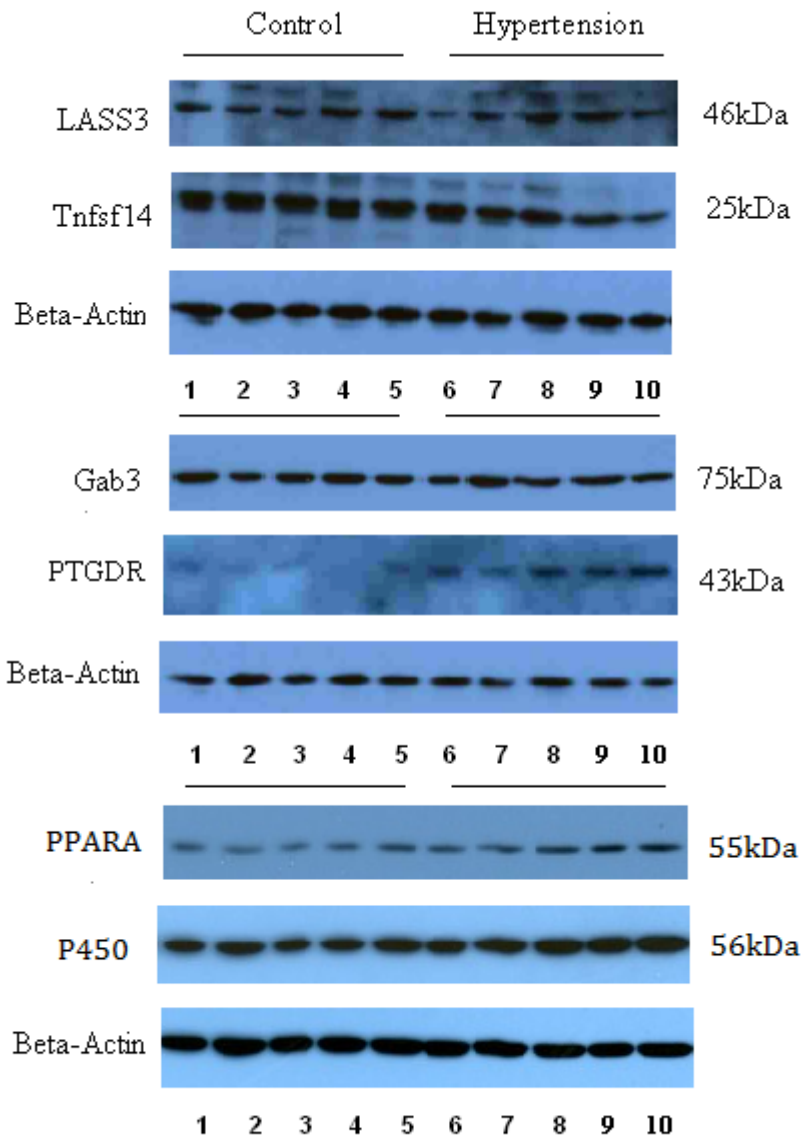
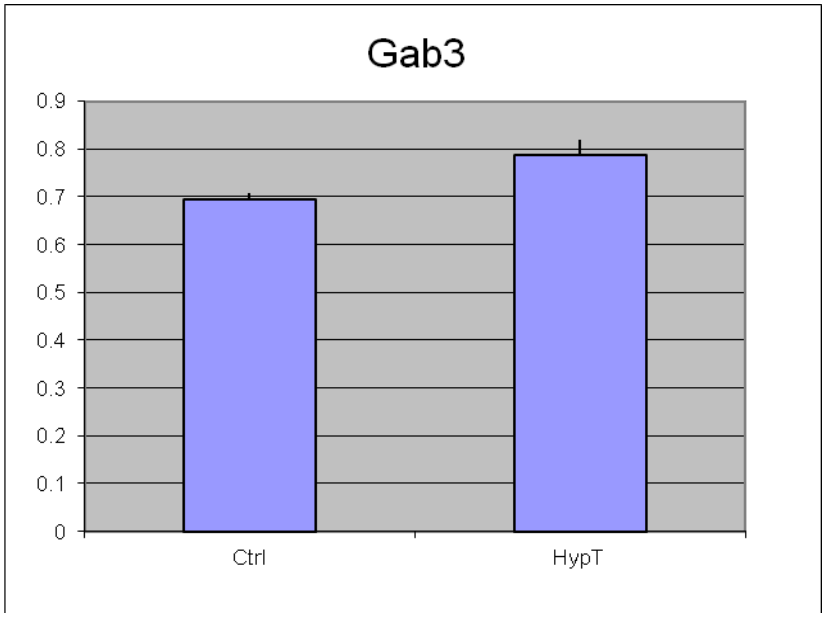


Fig 7. Western blot analysis of Gab3, Tnfsf14, Lass3, PTGDR, PPARA and P450 in comparison to Beta Actin. Sample size is 25µl. Data are plotted as mean ± S.D. and analyzed by Student's T-test. *P* < 0.05 indicates significant differences.



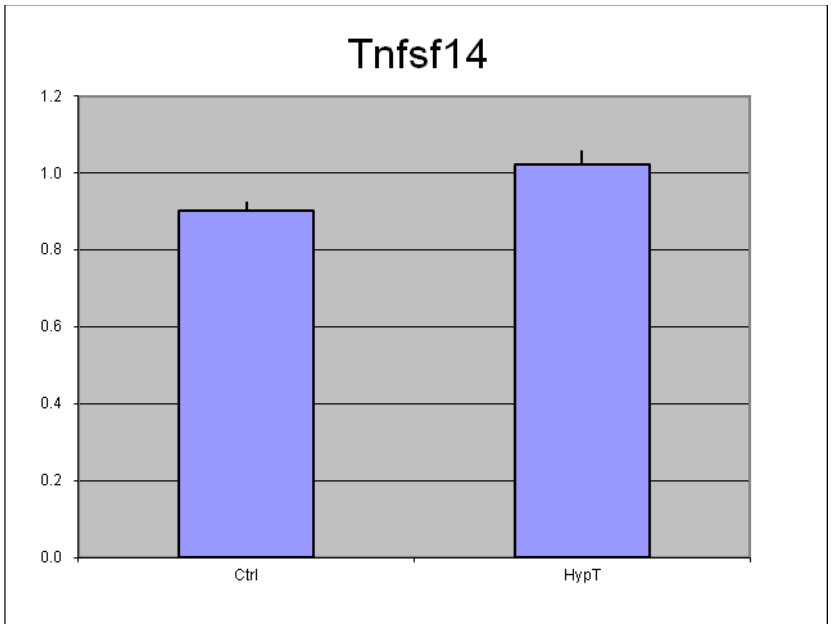
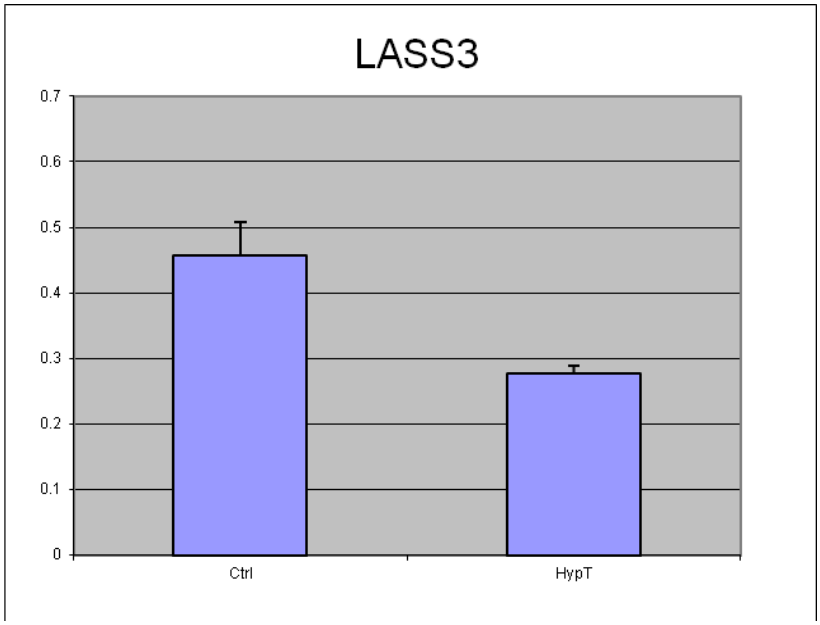


Fig 8a .Calculation of the gene expression of Gab3, Tnfsf14 in Western blot analysis.



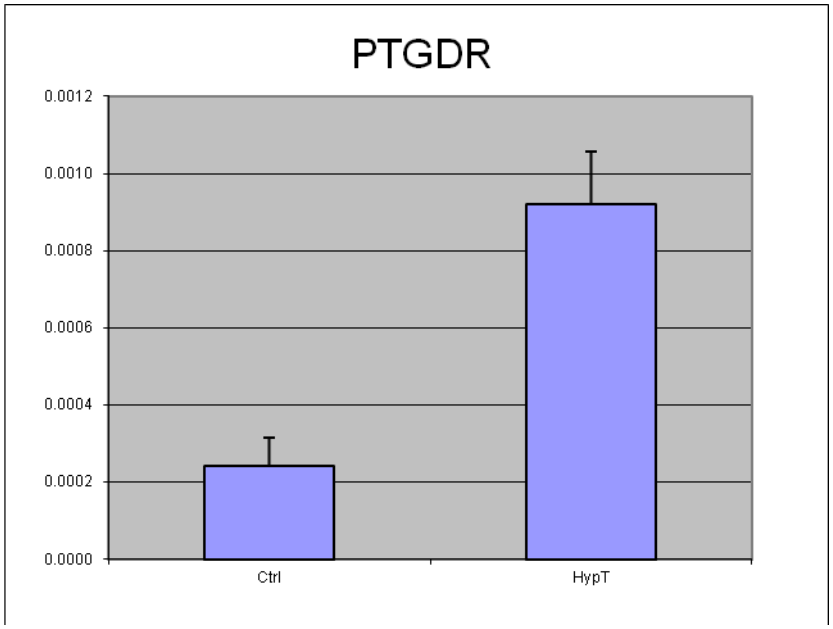
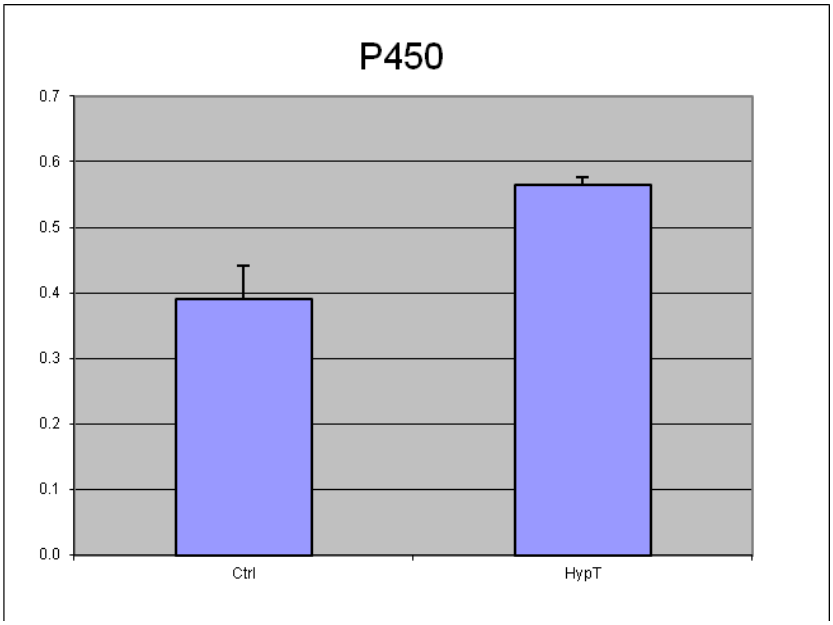


Fig 8b.Calculation of the gene expression of Lass3, PTGDR in Western blot analysis



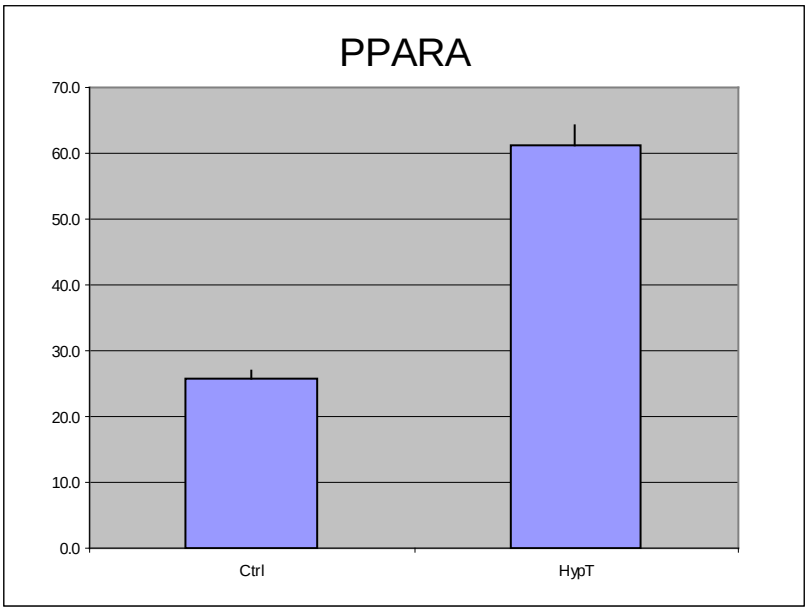


Fig 8c.Calculation of the gene expression of P450, PPARA in Western blot analysis.

Chapter 4: Discussion and Conclusion

4.1 Discussion

This study was carried out to examine the global gene expression in the rabbit MCA and FC upon consumption of high level of blood pressure as well as to further elucidate the pathways that might be important towards the contribution of arteriosclerosis. An initial step in the process of arteriosclerosis is endothelial dysfunction with endothelial macrophage adhesion, followed by their infiltration into the blood vessel wall. Endothelial dysfunction reduces nitric oxide (NO) production and leads to vasoconstriction.

Numbers of previous studies proved endothelial dysfunction is thought to be a key event in the development of atherosclerosis and predates clinically obvious vascular pathology (Abrams et al, 1997, Adams et al 1999). The reason is that endothelial dysfunction has strong relationship with reduced anticoagulant properties as well as increased adhesion molecule expression, chemokine and other cytokine release, and reactive oxygen species production from the endothelium, all of which play important roles in the development of atherosclerosis. In fact, endothelial dysfunction influenced significantly in predicting vascular events including stroke and heart attacks (Poredos et al.2006). Endothelial function testing may have great potential prognostic value for the detection of cardiovascular disease, but because of high price and difficult operation, currently the available tests are not wide accepted in clinical use.

Microarray analysis of the brain was used to identify differential gene expression in vessels and brain tissue from the rabbits at risk of hypertension. Although the weight changes and serum cholesterol level between treated and control groups were not significantly different, consumption of the 2-kidney, 1-clip Goldblatt hypertension model produced a marked increase in mean arterial pressure levels.

Acording to the primary scanning by Microarray results, we can find out some of them were repeatedly mentioned their importance by other related study. The canonical pathways analyses show PPARA is regulated by arachidonic acid, indomethacin and is found in the cytoplasm and nucleus of the cell. It appears to have roles in diseases such as hyperlipidemia, diabetes mellitus, hypercholesterolemia, Alzheimer's disease, carotid artery disease, coronary artery disease, atherosclerosis and hypertension. Previous study proved that PPARA is a transcription factor and a major regulator of lipid metabolism in the liver. PPARA is activated under nutrient-deficient conditions and is necessary for the process of ketogenesis, a key adaptive response to prolonged fasting (Kersten et al, 1999). Activation of PPARA promotes uptake, utilization, and catabolism of fatty acids by upregulation of genes involved in fatty acid transport and peroxisomal and mitochondrial fatty acid β-oxidation. PPARA is primarily activated through ligand binding. Synthetic ligands include the fibrate drugs, which are used to treat hyperlipidemia. An endogenous ligand has been identified as the phosphatidylcholine species 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (Chakravarthy et al, 2009). Endogenous activation of PPARA is dependent on the presence of fatty acid synthase.

P450 is located on the cell surface, Cytoplasm and its role in the cell involves respiration. It has been found to be associated with diseases such as drug toxicity, fibrosis, paralysis, hydrocephalus, hypertrophy, weight gain, and infarction.

TNF is an important gene that is regulated by several other genes, including lipopolysaccharide. It is found to be involved in several diseases and has several cellular functions and roles. This gene encodes a multifunctional proinflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. This cytokine is mainly secreted by macrophages. It can bind to, and thus functions through its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. This cytokine has been implicated in a variety of diseases, including autoimmune diseases, insulin resistance, and cancer. Knockout studies in mice also suggested the neuroprotective function of this cytokine (Nedospasov et al, 1986).

The protein encoded IL9 is a cytokine receptor that specifically mediates the biological effects of this gene. The functional IL9 receptor complex requires this protein as well as the interleukin 2 receptor, gamma (IL2RG), a common gamma subunit shared by the receptors of many different cytokines (Romero et al, 2010). The ligand binding of this receptor leads to the activation of various JAK kinases and STAT proteins, which connect to different biologic responses. This gene is

located at the pseudoautosomal regions of X and Y chromosomes. Genetic studies suggested an association of this gene with the development of asthma.

ASTN2 is another gene related to brain function. It encodes a protein that is expressed in the brain and may function in neuronal migration, based on functional studies of the related astrotactin 1 gene in human and mouse. A deletion at this locus has been associated with schizophrenia. Multiple transcript variants encoding different proteins have been found for this locus

In the FC, P450 was found to have the highest fold change (>24). This gene encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. The protein encoded by this gene localizes to the endoplasmic reticulum and its expression is induced by some polycyclic aromatic hydrocarbons (PAHs), some of which are found in cigarette smoke. The enzyme's endogenous substrate is unknown; however, it is able to metabolize some PAHs to carcinogenic intermediates. Other xenobiotic substrates for this enzyme include caffeine, aflatoxin B1, and acetaminophen. The transcript from this gene contains four Alu sequences flanked by direct repeats in the 3' untranslated region. In a system of purified components, human CYP1A2 protein increases metabolism of arachidonic acid (Choudhary *et al.* 2004)

Other genes that were found in FC were SELL and PTGDR. SELL encodes a cell surface adhesion molecule that belongs to a family of adhesion/homing

receptors. The encoded protein contains a C-type lectin-like domain, a calcium-binding epidermal growth factor-like domain, and two short complement-like repeats. The gene product is required for binding and subsequent rolling of leucocytes on endothelial cells, facilitating their migration into secondary lymphoid organs and inflammation sites. Single-nucleotide polymorphisms in this gene have been associated with various diseases including immunoglobulin a nephropathy. Selectin protein(s) is involved in adherence of platelets and endothelial cells that is increased by inflammation in organism (Juliano 2002). On the other hand, PTGDR is a G-protein-coupled receptor. It has been shown to function as a prostanoid DP receptor. The activity of this receptor is mainly mediated by G-S proteins that stimulate adenylate cyclase resulting in an elevation of intracellular cAMP and Ca2+. Knockout studies in mice suggest that the ligand of this receptor, prostaglandin D2 (PGD2), functions as a mast cell-derived mediator to trigger asthmatic responses. It also involved in eicosanoid signalling pathway.

Genes in common between MCA and FC were Gab3. This gene is a member of the GRB2-associated binding protein gene family. These proteins are scaffolding/docking proteins that are involved in several growth factor and cytokine signalling pathways, and they contain a pleckstrin homology domain, and bind SHP2 tyrosine phosphatase and GRB2 adapter protein. The protein encoded by this gene facilitates macrophage differentiation (Wolf *et al.* 2002). Two transcript variants encoding different isoforms have been found for this gene. Disease: AD (Li *et al.* 2008). Besides, Tnfs14 was also found with

common regulation both in MCA and FC. It is production and release of chemokines, cytokines (Blumberg *et al.* 2010, McInnes & Schett 2007). MMP (McInnes & Schett 2007),Found in neutrophil (Moore *et al.* 2001), macrophages (Green *et al.* 1994), endothelial cells. It has increase activation of endothelial cells (Hopkins 2007) and disruption of endothelial cells (Chen & Cosgriff 2000).

After detailed analysis by IPA system, all the selective genes were grouped by networks and locations. The gene maps clearly demonstrate the gene relation within the same network. They may have similar function or related to the same target or receptors. Here we picked up FC Network 1 map as the main study target, which include most of our interest genes such as Tnfsf14, PRLR, SELL, PTGDR and Gab3. Especially PPARA which standing in the center place of the network and tightly linked with many other functional genes, it might play an irreplaceable position and probably had strong influence on our target disease. The fact not only from the Real-Time PCR analysis but also the western blot detection both proved PPARA do has such a powerful influence: it keeps the top position of significance, followed by P450, PTGDR and other important genes, which means further research may pay more attention on the interaction between these groups of genes.

Since all theses involved genes are strongly related with stroke and other cardiovascular disease, or have correlative physiological function according to previous research, we can draw a conclusion that these genes will form a significant part in the formation of development of the diseases which are

mentioned above. The results from this study may support and provide evidences for the future research in exploring the principle of the stroke related diseases and contribute to discover the treatments for the stroke and hypertension patients.

4.2 Limitations and future study

The limitation of numbers of the animals and obvious physiological difference between individuals compromised of the data we collected from the samples. For example, the reproducibility one experimental animals is questionable by using the 2K1C method. For future study, an enlarged database is necessary to enhance the reliability of the results. If possible, more various models should be applied such as 1K1C method to form a meaningful comparison.

For the 1K1C model itself, it was first developed in 1934, which is quite a long time before today. Although it is a traditional and standard model, it still can be improved. Many articles reported that they combine the hypertension model together with high cholesterol food treatments, in order to discover the connections between hypertension and hyperlipidemia, which are always tightly related in clinical cases. And it is also our future plan for the next step.

During the step by step analysis, sometimes the same gene shows totally opposite direction of change. Take Gab3 as an example, it was upregulated in both microarray analysis and western blotting, however downregulated in RT-

PCR analysis. This situation may be caused by the low accuracy of large amount detection, the quality of the probes and antibodies, or the status of the samples. These can be avoided in the future. It is also conceivable that some of the genes have different expression according to changeable environment and locations. The possible presence of different splice forms or post-translational modifications of the protein targets may influence the discrepancy between observations from different methods. All of these can be very promising research direction to continue and develop this topic. Besides, there are still huge amount of gene selected by microarray, here we only picked 16 as our interest genes and more are waiting for the future project.

After all, there are more methods for more accurate detection could be applied in further research based on the results we got till now. Such as Immunohistochemistry and Electron Microscopy are the next step for discover more detailed information from the primary scanning results. Similar methods applied in other species of animals are also in the future plan of study.

4.3 Conclusion

This study applied microarray to detect the expression profile of various genes in the frontal cortex and middle cerebral artery that are involved and regulated in hypertension through the 2-kidney, 1-clip Goldblatt hypertension model. Genes were filtered step by step and subjected to the Ingenuity Pathway Analysis. Finally, four genes: PPARA, P450, PTGDR and LASS3 were selected as the most significant change after treatment. Most of them may have strong relationship based on the gene map and deeply involved in the target diseases. Further study around these genes may lead to a better understanding on the role of hypertension in stroke and other cardiovascular disease.

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